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Flavins Mediate Extracellular Electron Transfer in Gram-Positive *Bacillus megaterium* Strain LLD-1

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Abstract: The extracellular electron transfer (EET) mechanism of an isolated Gram-positive *Bacillus megaterium* strain (LLD-1), identified by 16S rRNA gene sequencing and physiological analysis, was investigated in the present study. The electrochemical activity of strain LLD-1 was confirmed by electrochemical *E-t* and amperometric *I-t* tests. Flavins in culture suspension from strain LLD-1 were further proved to be able to act as electron shuttles, strengthening the electron transfer from LLD-1 to the electrode. The output voltage and current output were increased 2.8 times and 3.7 times, respectively, by adding 100 nM exogenous flavins into microbial fuel cells inoculated with LLD-1. Electricity generation by LLD-1 from different carbon sources can be enhanced by adding 100 nM exogenous flavins. This study indicated that flavins were essential to the EET process of the Gram-positive strain LLD-1. Furthermore, a putative EET model for *B. megaterium* strain LLD-1 and even for Gram-positive bacteria was proposed.

Keywords: extracellular electron transfer; microbial fuel cells; flavin; electron shuttle; *Bacillus megaterium*

1. Introduction

Electrochemically active microorganisms (EAMs) are species that can transport electrons from cells to extracellular electron acceptors such as minerals, contaminants and electrodes, and the electron transfer processes are defined as extracellular electron transfer (EET) [1]. Therefore, investigations into EAMs and their EET mechanisms are of vital importance to understanding various biogeochemical processes and to developing technologies for contaminant degradation coupled with energy production (such as bioelectrochemical systems) [2].

In recent years, more than 100 EAMs have been isolated or identified, covering a wide range of genetic groups, and Gram-negative *Proteobacteria* is the most abundant phylum [3, 4]. However, there is still limited knowledge on the EET mechanisms, as the proposed EET pathways are mainly based on studies targeting gram-negative *Shewanella* spp. and *Geobacter* spp. So far, two EET mechanisms have been proposed for EAMs, a direct electron transfer pathway via outer membrane *c*-type cytochrome proteins [5-7] or extended conductive pili/nanowires [8, 9] and an indirect electron transfer mode via electron shuttles such as flavins [10, 11].

The electron-shuttling compounds, either self-produced by EAMs [11, 12] or exogenetic, can assist in microbial energy metabolism by facilitating EET processes. These water soluble electron shuttles can also enhance the bioremediation of contaminated environments by facilitating microbial contaminant transformations that are physically/spatially unavailable to microbes [13]. The cell walls of Gram-negative bacteria, i.e., the outer membranes, are much thinner (typically 10 nm) than those of Gram-positive bacteria (typically 30 to 80 nm). It has been noted that the structure and thickness of the cell

membrane/wall are very important to microbial EET processes [14-16]. However, few studies have focused on gram-positive bacteria even though they spread widely in various environments [14-23]. These studies have focused on their current production in MFCs by direct electron transfer [20,21] or through reducing exogenous electron mediators (humic acids, anthraquinone-2,6-disulfonate, etc.) to generate current indirectly [18] and their ability to reduce Fe(III) oxides [15,22]. In other words, endogenous mediators have rarely been reported/mentioned in proposed EET mechanisms for Gram-positive bacteria. S-layer associated *c*-type cytochromes (SLC) have been found on the cell wall surface of Gram-positive bacteria by Chang et al. [15]. Whether and how endogenous mediators work on SLC are still unsolved questions, even if that endogenous mediators are proved to be secreted by Gram-positive bacteria.

Bacillus megaterium (*B. megaterium*), one kind of Gram-positive industrial bacterium, is used extensively for producing poly-hydroxybutyric acid, which is an organic polymer with potential commercial applications as a biodegradable thermo-plastic biomaterial [24]. The species is also a potential agent for biocontrol of diseases in tea plants and can be used for effective degradation of organophosphorus pesticides [25], dichloroanilines, monosultap and other hazardous cargos [26]. Cheung and Gu found that *B. megaterium* is able to reduce the toxicity of some pollutants, e.g., transforming Cr(VI) to Cr(III) [27]. However, the mechanisms by which *B. megaterium* interacts with and transforms heavy metallic salts or other contaminants are still unknown. Coman *et al.* reported that the electrochemical communication between Gram-positive *Bacillus* spp. strains and electrodes can be enhanced by osmium redox polymer electrodes [28]. Furthermore, long-range electron transfer via mediators through mass diffusion is beneficial to degrading pollutants in wastewater. Thus, investigation into EET mechanisms via the endogenous mediator of *B. megaterium* is essential for understanding the interactions at the SLC/electrode interface.

In our previous work, riboflavin was found in gram-positive *Bacillus* sp. WS-XY1 and yeast *Pichia stipites* [29]. In the present study, we report a new EAM, *B. megaterium* strain LLD-1, which was isolated from the anode of a microbial fuel cell (MFC) and identified by 16S rRNA gene sequencing technique coupled with physiological/biochemical identification methods. All soluble flavins related to EET mechanisms were measured based on cyclic voltammetry (CV), differential pulse voltammetry (DPV), chronoamperometric tests and high-performance liquid chromatography (HPLC). Moreover, an electron-transfer model for *B. megaterium* strain LLD-1 and gram-positive bacteria were comprehensively discussed and proposed.

2 Experimental Section

2.1. Bacterial isolation and culture

A MFC was constructed as shown in Scheme S1. The anode chamber was inoculated with activated sludge from JiMei wastewater treatment plant in Xiamen City and fed with sodium acetate (analytical reagent) of 8.93 mM (i.e., $1 \text{ g} \cdot \text{L}^{-1}$) and 0.10 M phosphate buffer solution (PBS, KH_2PO_4 of 0.05 M, K_2HPO_4 of 0.05 M, pH 7.0, analytical reagent). After the MFC can stably output current of approximately 0.5 mA with 1000Ω external resistance, biofilm was washed down from one piece of the anode ($1 \times 1 \text{ cm}$) and dispersed in 50 mL sterilized water. Then, bacteria were isolated following the method described in our previous study [30].

All experimental supplies were sterilized at 121°C for 20 min before inoculation. Here, *B. megaterium* strain LLD-1 (indexed as LLD-1) was cultured in fresh sterilized peptone-glucose medium ($20 \text{ g} \cdot \text{L}^{-1}$ peptone, $20 \text{ g} \cdot \text{L}^{-1}$ glucose, pH 7.0, Biological reagents) and grown aerobically with a slight shake of 150 rpm at 32°C . The physiological and biochemical tests were performed when the optical density of the cell suspension at 600 nm ($\text{OD}_{600\text{nm}}$) reached 1.0.

2.2. DNA extraction, the 16S rRNA gene sequencing and physiological and biochemical test

Total genomic DNA was extracted from bacterial cells grown in YPD medium (20 g·L⁻¹ peptone, 20 g·L⁻¹ glucose, 10 g·L⁻¹ yeast extract, pH 7.0) using a previously reported method PL [31]. A 1.5-kb fragment of the 16S rRNA gene was amplified by PCR on a MasterCycler gradient PCR apparatus (Eppendorf, Germany). The primer sequences used for the amplification were 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5'-TAC GGC TAC CTT GTT ACG ACT T-3' for forward and reverse primer, respectively. After purification the PCR product was ligated into a pMD19-T vector (Takara, China) and then transformed into *Escherichia coli* competent cells to isolate plasmids containing the insert for sequencing (Shanghai Majorbio Bio-Pharm Technology Co., Ltd, China). The homology of the 16S rRNA partial gene sequence was compared with the existing sequences available in the data bank using a BLAST search. The sequence alignment was carried out using Clustal W (version 1.83) of NCBI and the phylogenetic tree was constructed by MEGA software (version 5.2) using the Neighbour Joining method.

Biochemical tests based on the API 50 CHB gallery (Biomérieux, France) were used to investigate the fermentation of 49 sugars (Biological reagents) according to the manufacturer's instructions, including D-cellobiose, glycerol, D-maltose, erythritol, D-lactose, D-arabinose, D-melibiose, L-arabinose, D-sucrose, D-ribose, D-trehalose, D-xylose, inulin, L-xylose, D-melezitose, D-adonitol, D-raffinose, D-galactose, Methyl-βD-mannopyranoside, starch, glycogen, D-glucose, xylitol, D-fructose, gentiobiose, D-mannose, D-turanose, L-sorbose, D-lyxose, L-rhamnose, D-tagatose, dulcitol, D-fucose, inositol, L-fucose, D-mannitol, D-arabitol, D-sorbitol, L-arabitol, methyl-αD-mannopyranoside, 5KG potassium gluconate, methyl-αD-glucopyranoside, 2KG potassium gluconate, N-Acetyl-glucosamine, esculin/ferrous citrate, amygdaline, salicin, arbutin and gluconate. Other physiological and biochemical tests were performed according to Bergey's Manual of Determinative Bacteriology.

2.3. Morphological characterization

For scanning electron microscopy (SEM) imaging, bacterial cells were carefully collected by centrifugation at 5000 rpm for 5 min, washed three times with 0.10 M PBS (pH 7.4) and then immersed in 2.5% (v/v) glutaraldehyde with 0.10 M PBS for 4 h at 4 °C [18]. The cells subsequently were incubated in 0.1 M PBS for 1 h and rinsed with distilled water to remove and dissolve salts, followed by dehydration with 50%, 75%, 90%, 95%, and 100% (v/v) ethanol solution each for 5 min. The cells were finally dried in a drying oven at 60 °C for 12 h. The surface morphology of the bacteria was observed by S-4800 SEM (Hitachi, Tokyo, Japan, operated at 5 kV).

2.4. Electrochemical characterization

CV and DPV (potential increment: 4 mV; amplitude: 50 mV; pulse width: 0.06 s; pulse period: 0.5 s) experiments were carried out in a three-electrode system to investigate the electrochemical redox activity of strain LLD-1 by an electrochemical workstation (CHI660D, China). A glassy carbon (GC) electrode (3 mm diameter) was used as the working electrode while a platinum wire and Ag/AgCl (sat. KCl) served as counter and reference electrode, respectively. Bacterial cells were harvested with an optical density at OD_{600nm} of 1.0 in stationary phase. Cells were collected by centrifugation at 5000 rpm for 5 min and then washed three times with 0.10 M PBS (pH 7.0). Precipitated cells (5 µL) were directly transferred onto the GC electrode surface for electrochemical measurements referred to a bare GC without cells.

Amperometric *I*-t curves and *E*-t curves were performed in 125 mL two-chamber MFCs to evaluate the contribution of 100 nM flavins (33.3 nM each for FAD, FMN and RF) to EET efficiency as indicated by current generation and output voltage. Glucose culture medium (20 g·L⁻¹) with or without cells was set as controls. Strain LLD-1 at OD_{600nm} of 1.0 was cultivated in peptone-glucose medium (20 g·L⁻¹ peptone, 20 g·L⁻¹ glucose, pH 7.0) on a GC

electrode poised at +400 mV (vs. Ag/AgCl in sat. KCl) with CHI1000B (China) in a constant temperature incubator. All the solutions were deoxygenated by purging with nitrogen gas for 20 min before the test and maintained under nitrogen atmosphere during the electrochemical measurements [32]. All the electrochemical tests were in triplicate.

2.5. Quantitative analysis of flavins by HPLC

Commercially available FAD (Sigma), FMN (Sigma) and RF (Sigma), serving as reference standards in the HPLC analysis, were dissolved in water. The excitation/emission fluorescence spectra of these flavins were collected to optimize the detection condition of HPLC shown in Figure S1. The characteristic fluorescence absorptive bands of flavins (RF-551, Shimadzu, Duisburg, Germany) indicated that the fluorescence detection wavelength could be set at 468/525 nm (excitation/emission). Culture suspensions of 5 mL in peptone-glucose medium collected at different culture times were centrifuged and filtrated with 0.22- μ m membrane filters. Then, the series of 10 μ L supernatant samples were injected into an HPLC system (Agilent 1200, USA) consisting of an online degasser (DG-2080-53), a gradient former (LG-1580-02), a PU-980 pump and an AS-1555 autosampler. It was performed at a flow rate of 1 mL/min on a reversed-phase C18 column (Eclipse Plus C18, 4.6 \times 250 mm, 5 μ m) at 30 °C. The mobile phase consisted of methanol (analytical grade) (phase A) and water-650 mM glacial acetic acid (analytical grade) (phase B). The gradient elution protocol was as follows: 91% phase B for 8 min, 91% phase B to 11% phase B in 8 min, 11% phase B for 3 min, 11% phase B to 91% phase B in 11.1 min, and 91% phase B for 5 min.

2.6. MFC construction and operation conditions

The configuration of the MFCs is shown in Scheme S1. The cylindrical anode chamber 8 cm in diameter was filled with 100 mL of PBS basal medium with a mineral and vitamin

mixed as described by Baron *et al.* while the cathode chamber was filled with 50 mM $K_3[FeCN_6]$ (analytical grade) and 50 mM PBS (pH 7.0) [33, 34]. The two chambers were separated by a cation exchange membrane (Zhejiang Qianqiu Group Co. Ltd., China) as previously reported [35, 36]. Graphite felt (Haoshi Carbon Fiber Co. Ltd., China) electrodes approximately 2×2 cm were used as anodic and cathodic electrodes. The two chambers were connected with Ti wire and the resistor was 1000 Ω . Before the inoculation, all the MFCs were sterilized by autoclaving at 121 °C for 20 min. To evaluate the effects of carbon source on power generation, glucose, glycerol, acetate, sucrose, and lactose (each 0.1 M) prepared with 50 mM PBS (pH 7.0) were used individually as the sole electron donor to culture cells in fed-batch mode (30±2 °C). All the solutions used in the anode chamber were deoxygenated by purging with nitrogen gas for 30 min. Ten millilitres of strain LLD-1 at the exponential phase was inoculated into the MFCs (experiment groups), with no bacterial inoculation in the control group. Then, the chambers were sealed with a rubber stopper as shown in Scheme S1. Moreover, the effects of exogenous flavins (100 nM) on the power generation of MFCs were investigated in the experiment groups. The cell voltage of MFCs was recorded every 5 min with a digital multimeter (Keithley Instruments, Inc., USA). All the MFCs were operated under anaerobic conditions.

2.7. MFC performance measurement and analysis

The performance of MFCs is commonly characterized by polarization curves, which are obtained by varying the external resistance in a fuel cell test system (Maccor, USA). Power density (P) was determined as follows [34]:

$$P = U \times I / A \quad (1)$$

where U and I are the cell voltage and the corresponding current, respectively. A is the projected surface area of graphite felt.

3. Results and Discussion

3.1. Sequencing and phylogenetic analysis

A bacterial strain designated LLD-1 was isolated from the anodic biofilm of an MFC fed with acetate sodium. For 16S rRNA gene analysis, strain LLD-1 was cultured on a Luria-Bertani plate, and five clones were randomly selected for DNA extraction and Sanger sequencing. The sequencing provided a unique 16S rRNA gene sequence, and phylogenetic analysis suggested that the isolated LLD-1 is a member of the genus *Bacillus* (Figure 1). The 16S rRNA gene sequence of LLD-1 shares similarity of 99% to the gene sequence of *Bacillus megaterium* strain HNS88 (GenBank accession no KF933685.1).

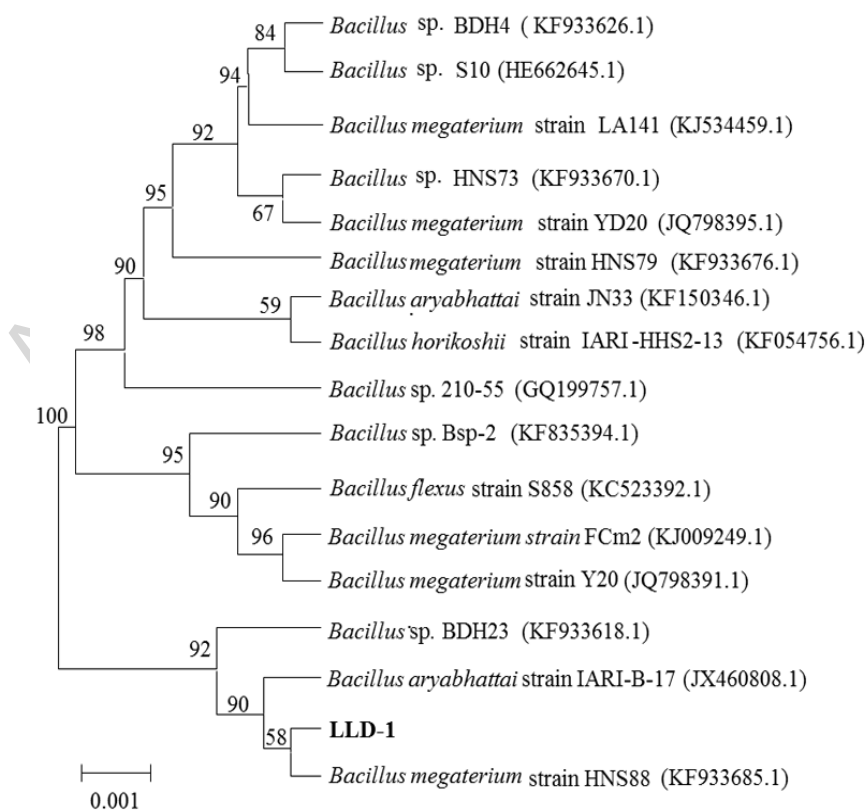


Figure 1. Phylogenetic tree of stain LLD-1 and closely related species based on 16S rRNA sequences. The tree was constructed using the neighbour-joining method.

From the physiological and biochemical characterization results, we found that strain LLD-1 is a Gram-positive bacterium that can generate spores in its later growth stage. Catalase is produced, and starch can be hydrolysed. It can grow between 4 °C and 40 °C, with an optimal temperature of approximately 37 °C. Strain LLD-1 can reduce nitrate to nitrite. Gelatin and aesculin can be hydrolysed. NaCl is tolerated up to 7% (w/v). In the API 50CHB gallery, the results of acid production from carbohydrates are shown in Table S1. The expression of phenotypic traits was in accord with *Bacillus megaterium*.

3.2. Morphological Characterization

From SEM morphological images of LLD-1 (Figure 2), the strain can be easily recognized as its colonies are found to be big (diameter of approximately 5 mm), lucid, smooth and irregular. The bacteria prefer to gather together and form a long chain as reference reported [36], where the cells were straight rod-shaped 2-5 µm long and 1 µm wide.

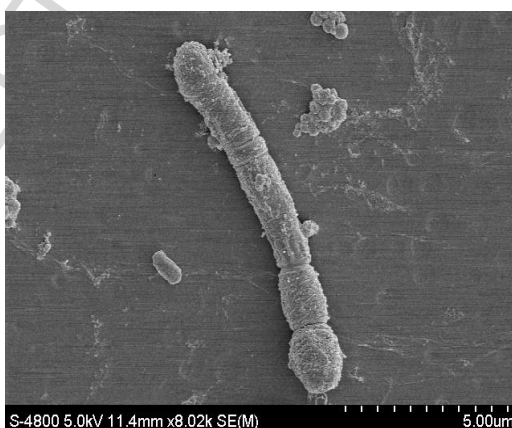


Figure 2. Scanning electron microscopy images of *Bacillus megaterium* strain LLD-1.

3.3. Electrochemical Characterization

Four redox peaks are found with potential at E_p of +100 / -120 mV (group I) and E_p of -395 / -425 mV (group II) when LLD-1 is loaded on a bare GC electrode (results from DPV

measurement are shown as insets) (Figure 3a). No redox peak was found in the control with 0.10 M PBS.

According to previous reports, *Shewanella oneidensis* releases low concentrations of flavins, i.e., flavin mononucleotide (FMN) and riboflavin (RF), into culture medium to act as shuttles for EET processes [10, 37]. FMN and RF show redox potentials similar to the group II redox peaks for LLD-1. The CVs and DPVs of culture supernatant were therefore collected (Figure 3b), and a pair of reversible redox peaks at similar potentials was clearly shown in the CV/DPV curves. We thus inferred that the peaks of group II could be ascribed to dissolved flavins (Figure S2) [6], and the peaks of group I may be assigned to *c*-type cytochromes in the bacterial cell membrane proteins. Then, RF standard solutions at final concentrations of 50 and 100 nM were separately added into the culture supernatant to determine the peak assignment from CV and DPV measurements (Figure 3b). It can be found that the intensity of redox peaks increased with the increasing amount of RF. Therefore, it can be proposed that flavins were present in the LLD-1 culture supernatant.

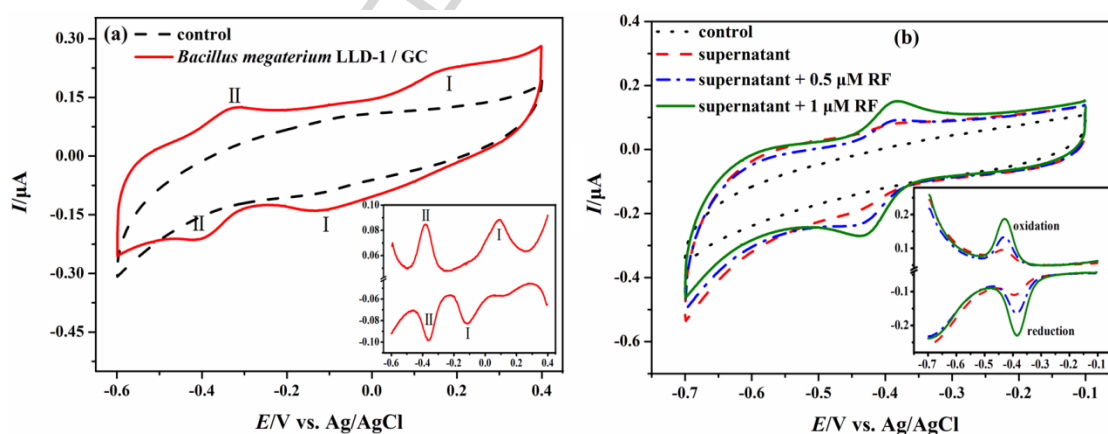


Figure 3. (a) CVs for a bare GC electrode (dot line) and *B. megaterium* LLD-1 on a GC electrode (solid line) in 0.10 M phosphate buffer solution (PBS, KH_2PO_4 of 0.05 M, K_2HPO_4 of 0.05 M, pH adjusted to 7.0); (b) CVs for the bare GC electrode in culture supernatant in the presence of 0 (red dash line), 50 (blue dash dot line), or 100 nM RF (green solid line) and the

control in 0.10 M PBS (pH 7.0) (black dot line). Scan rate for all CVs is 10 mV s^{-1} . The corresponding DPV measurements (potential increment: 4 mV; amplitude: 50 mV; pulse width: 0.06 s; pulse period: 0.5 s) are shown as insets.

3.4. HPLC analysis of flavins in LLD-1 culture medium

Flavin standards, i.e., flavin adenine dinucleotide (FAD), FMN and RF, can be well separated in the mixture sample using HPLC (Figure 4a). The peaks successively eluted at 7.3, 8.0 and 8.6 min were attributed to FAD, FMN and RF in comparison with reference standards.

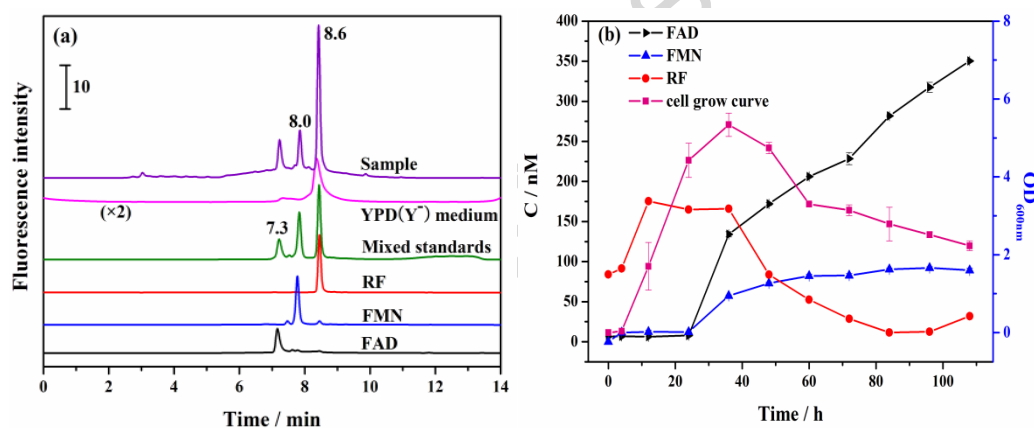


Figure 4. (a) HPLC chromatograms for single and mixed standard solutions and samples collected at 36 h of cell culturing; (b) Concentration curves of FAD, FMN and RF in culture medium of LLD-1 versus different culture times coupled with cell growth curve. Error bars in (b) represent triplicate experiments.

LLD-1 was inoculated into 100 mL of sterilized peptone-glucose broth in 250 mL flasks at 30 °C. The concentration curves of flavins in culture medium coupled with cell growth are plotted in Figure 4b, wherein 84 nM RF and 6.5 nM FAD were detected in initial sterile peptone-glucose medium, respectively. It can be found that RF increased sharply within 12 h, i.e., the log growth phase, and then was maintained at a stable concentration of approximately 160 nM before 36 h.

Since FAD is mainly involved in intercellular metabolism, an increase of FAD can be used as an indicator for cell lysis. In the present study, FAD began to increase at 24 h. When the culturing time was at 36 h, i.e., the initial time point of growth phase decline, cell lysis became notable and RF decreased quickly accompanied by a significant increase of FAD and FMN. This result therefore indicated that RF detected in the log growth phase came from initiative secretion by bacteria rather than cell lysis. In comparison, RF and FMN were reported to increase throughout the whole cultivation period of *Shewanella* strains, but FAD was only detected after cell lysis [36]. Furthermore, FAD and FMN from *Shewanella* strains are chemically unstable and transform to RF very fast [10]. In this study, we thought this transformation did not occur as RF continuously decreased from 36 to 96 h and FMN and FAD continuously increased during the same period. We further investigated the effect of RF on cell growth (Figure S3). The addition of low concentration exogenous RF slightly promoted cell growth in the adaptation period but inhibited cell growth in the logarithmic phase and decline phase.

3.5. Effect of flavins on power generation and EET efficiency

To determine whether the LLD-1 strain can respond to electron donors and how the extra-flavins affect current output or power generation, $E-t$ and amperometric $I-t$ experiments in MFCs were conducted to confirm our points.

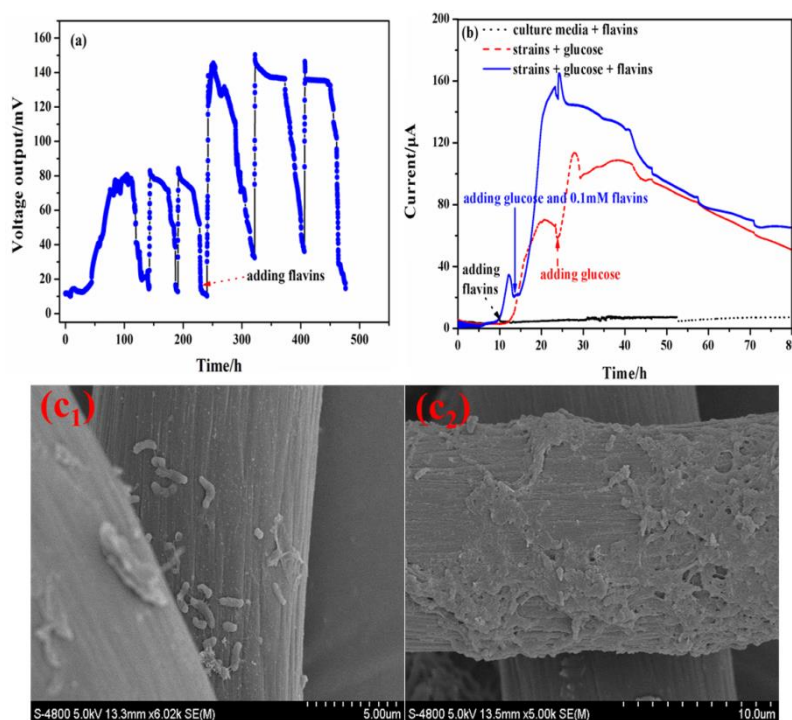


Figure 5. (a) $E-t$ curve showing the effect of exogenous flavins on the output voltage of MFCs cultured with glycerol; (b) Amperometric $I-t$ curve showing the response of LLD-1 to glucose without (dashed line) or with (solid line) 100 nM flavin mix (33.3 nM each for FAD, FMN and RF) comparing to glucose ($20 \text{ g}\cdot\text{L}^{-1}$) culture medium with addition of flavin mix (dot line), and the addition points are indicated by arrows; (c) SEM images of LLD-1 and anodic graphite felt when MFCs were fed by glycerol medium without (c_1) or with (c_2) extra flavins. All experiments are in triplicate.

Figure 5a shows the $E-t$ curve of an MFC where LLD-1 was cultured with the sole carbon source of glycerol. The output voltage increased slowly within 50 h for the reason that the cell was at the adaptive phase, then it rapidly reached its maximum level and finally decreased to approximately 20 mV due to inadequate supplies of glycerol. To start a new cycle, glycerol was added into the culture at the point of approximately 12 mV. The result showed that current can be generated from glycerol-fed LLD-1, i.e., LLD-1 cells can respire glycerol and transport electrons to an extracellular electrode. The stable maximum output voltage of the MFC was approximately 85 mV in the early three cycles and then suddenly increased to approximately 148 mV with the addition of 100 nM exogenous flavins. In this

case, a 74% increment of the voltage generation was achieved with exogenous flavins. Corresponding SEM images of strain LLD-1 on anodic graphite felt are shown in Figure 5c. Comparing to biofilm formed without exogenous flavins (Figure 5c₁), more cells attached onto the electrode surface in the presence of the exogenous flavin mix (Figure 5c₂). The result indicated that flavins not only promote EET processes but also enhance the thickness of biofilm.

The effects of flavins on EET processes were also studied by amperometric $I-t$ in two-chamber MFCs (Figure 5b). The current barely increased when adding 100 nM flavin mix into MFCs without LLD-1 (i.e., the control). The current in MFCs with LLD-1 greatly increased from approximately 60 to 115 μA from glucose addition, indicating current production and electron transfer from cells to electrode by LLD-1. A sharp rise of current from 20 to 160 μA was observed after adding glucose and 100 nM flavins. The result further indicated that 85 μA of current generation can be enhanced in the presence of exogenous flavins.

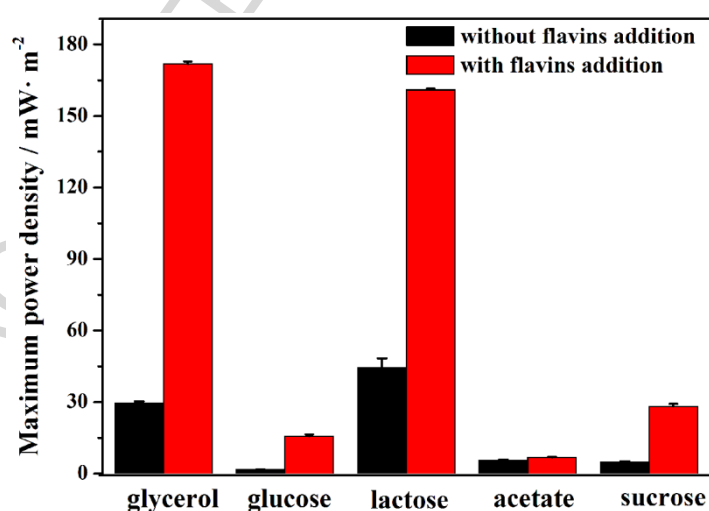


Figure 6. Maximum power density of MFCs boosted in different carbon sources without and with extra flavins extracted from polarization curves.

The power output of MFCs using different carbon sources also indicated that flavins are able to mediate the EET processes of LLD-1. As shown in Figure 6, MFCs fed with lactose generated the highest maximum power density of 44 mW m^{-2} . Power output density of 29 and 5.7 mW m^{-2} was collected by glycerol- and acetate-fed MFCs, respectively. On the other side, the lowest power output density of 1.6 mW m^{-2} was produced by glucose-fed MFCs while sucrose-fed MFCs output a power of 4.6 mW m^{-2} . The result indicated that electricity generation in MFCs with LLD-1 dramatically depends on the type of carbon source.

Different substrates coupled with 100 nM exogenous flavin mix were added into MFCs when the output voltage decreased to approximately 12 mV after the third batch, and a dramatic increase of power density was observed in most MFCs. The maximum power density of 170 mW m^{-2} was achieved in glycerol-fed MFCs, which was 4.6 times higher than that without flavin addition. For lactose-fed MFCs, 160 mW m^{-2} was achieved with a 3-fold increment. Similar increases of power density were also observed in glucose- and sucrose-fed MFCs, although the power density in these MFCs was still relatively low. However, no obvious increase of power output occurred in the non-fermentable acetate-fed MFCs.

Normally, *Bacillus* spp. usually produce acetoin, lactate and acetate as the metabolic products as a consequence of mismatched glycolytic and tricarboxylic acid cycle capacities when glucose is used as carbon source [38,39]. No related works have reported whether acetate can be used as the sole carbon source by *Bacillus* spp. It was interesting to find that current was generated in acetate-fed MFCs with *B. megaterium* strain LLD-1. The result indicated that acetate as a metabolic product of the glucose cycle can be respired by LLD-1 and enter into its special metabolism cycle to produce current.

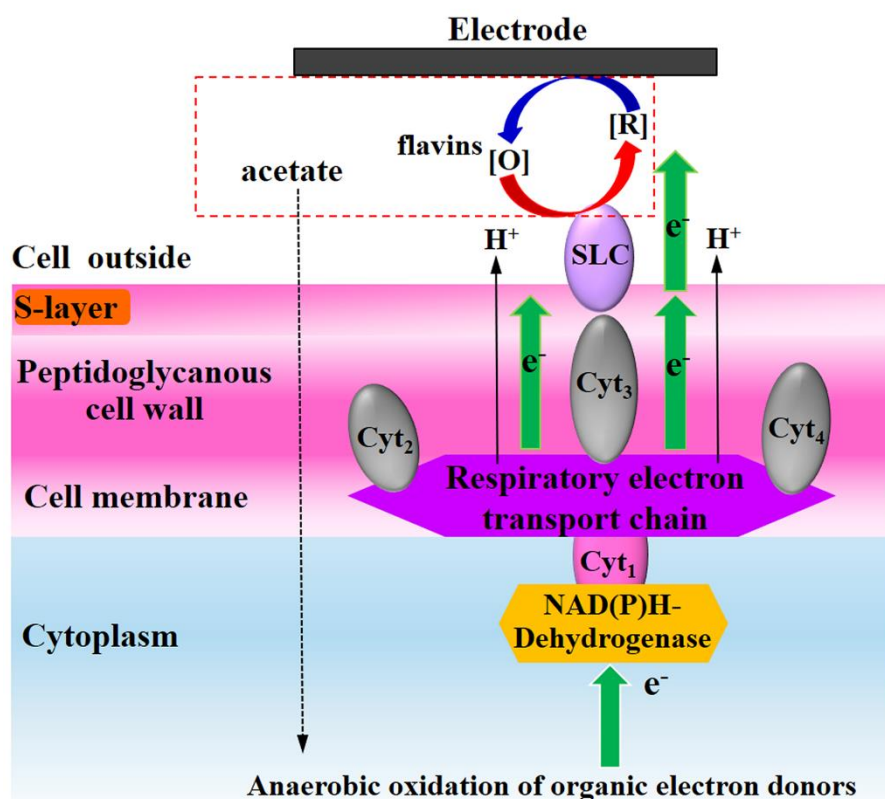
Based on previous reports on the EET mechanisms of electrochemically active

Gram-positive bacteria [14-23, 28, 29] and the results from this study, we propose a putative EET model for *B. megaterium* strain LLD-1 and even for Gram-positive bacteria as follows (Schematic 1):

1) Reduction of insoluble or soluble Fe(III) oxides in most Gram-positive bacteria involves the respiratory electron transport chain shown in Schematic 1. Electrons obtained from the oxidation of organic electron donors (such as glucose and glycerol) enter the chain via NAD(P)H-dehydrogenase, the quinone pool and then transmembrane proteins (indexed as cytochrome C_1). SLC on the cell wall surface can transfer electrons from membrane-bound cytochrome C_3 in the respiratory electron transport chain to insoluble Fe(III) oxides, which has been reported in *Clostridium butyricum* [15, 22].

2) Direct electron transfer between Gram-positive bacteria and electrode mainly passes through membrane-bound *c*-type cytochromes. Pili are only found in *Clostridium butyricum* using ferrihydrite as a sole electron acceptor.

3) Exogenous electron mediators are also employed in MFCs. However, some intermediates, especially those uncharacterized but important to EET processes, are neglected due to their complex underlying mechanisms. Although flavins are proved to be able to mediate the EET processes in *Bacillus* spp. in this study, it is unclear where the reactive/bonding sites are for flavins on the S-layer and how flavins mediate the EET process at the molecular level.



Schematic 1. Putative EET model for Gram-positive bacteria: where SLC is S-layer associated *c*-type cytochromes on the cell wall surface [22]; Cyt₁ is membrane *c*-type cytochromes, and Cyt₂, Cyt₃, Cyt₄ are all uncertain membrane-bound Fe(III)-reducing *c*-type cytochromes [16]; the EET pathway via flavins and acetate metabolism for *B. megaterium* spp. is depicted in the red-dotted frame.

4 Conclusions

The EET mechanism of a newly isolated gram-positive strain, *Bacillus megaterium* LLD-1, was studied in the present work. RF secreted by *B. megaterium* LLD-1 in supernatant occupied the largest proportion of flavins and strongly correlated with bacterial growth. A 2.8-fold increment of output voltage was achieved by adding 100 nM exogenous flavin mix into LLD-1 inoculated MFCs, which can efficiently utilize electron donors of lactose (3-fold increment) and glycerol (4.6-fold increment) to generate more power. The results suggest a putative EET model for the *B. megaterium* strain and even for

Gram-positive bacteria. There are still many unknown issues present in the EET processes for Gram-positive bacteria, and more attention may be paid to the S-layer associated *c*-type cytochromes and the functions of electron-carrying mediators. However, it is worth mentioning that acetate, as a metabolic product from glucose, can be respired by LLD-1. In a word, this study expanded our understanding of the roles of Gram-positive strains in biogeochemical and bioelectrical processes based on flavin-mediated EET.

Supplementary Materials are available online.

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Figure legends

Figure 1. Phylogenetic tree of stain LLD-1 and closely related species based on 16S rRNA sequences. The tree was constructed using the neighbour-joining method.

Figure 2. Scanning electron microscopy images of *Bacillus megaterium* strain LLD-1.

Figure 3. (a) CVs for a bare GC electrode (dot line) and *B. megaterium* LLD-1 on a GC electrode (solid line) in 0.10 M phosphate buffer solution (PBS, KH_2PO_4 of 0.05 M, K_2HPO_4 of 0.05 M, pH adjusted to 7.0); (b) CVs for the bare GC electrode in culture supernatant in the presence of 0 (red dash line), 50 (blue dash dot line), or 100 nM RF (green solid line) and the control in 0.10 M PBS (pH 7.0) (black dot line). Scan rate for all CVs is 10 mV s^{-1} . The corresponding DPV measurements (potential increment: 4 mV; amplitude: 50 mV; pulse width: 0.06 s; pulse period: 0.5 s) are shown as insets.

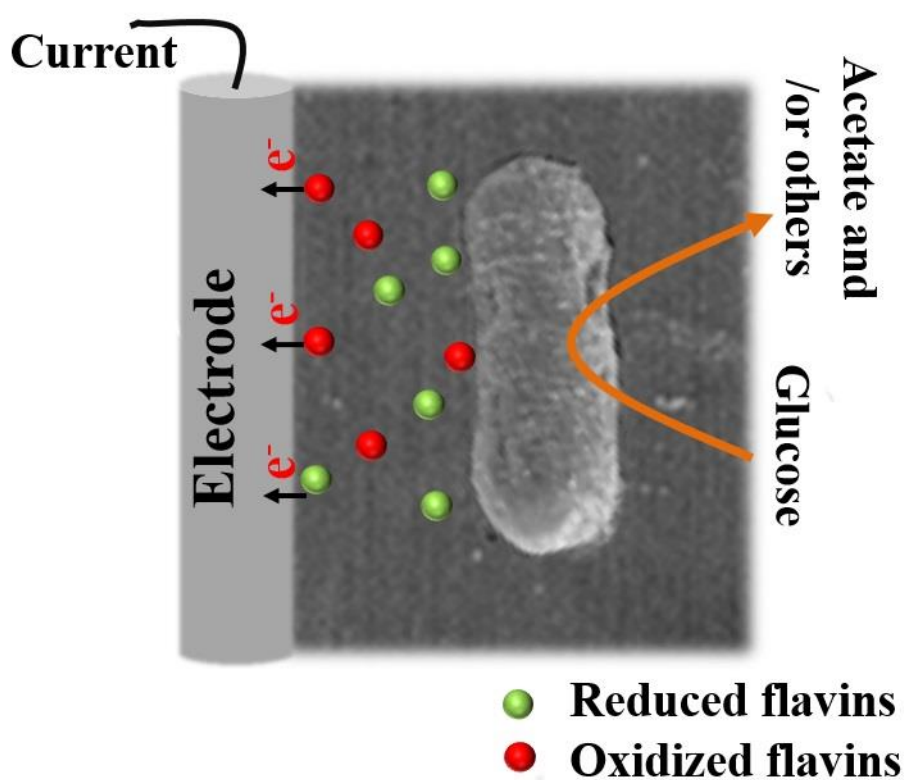
Figure 4. (a) HPLC chromatograms for single and mixed standard solutions and samples collected at 36 h of cell culturing; (b) Concentration curves of FAD, FMN and RF in culture medium of LLD-1 versus different culture times coupled with cell growth curve. Error bars in (b) represent triplicate experiments.

Figure 5. (a) $E-t$ curve showing the effect of exogenous flavins on the output voltage of MFCs cultured with glycerol; (b) Amperometric $I-t$ curve showing the response of LLD-1 to glucose without (dashed line) or with (solid line) 100 nM flavin mix (33.3 nM each for FAD, FMN and RF) comparing to glucose ($20 \text{ g}\cdot\text{L}^{-1}$) culture medium with addition of flavin mix (dot line), and the addition points are indicated by arrows; (c) SEM images of LLD-1 and anodic graphite felt when MFCs were fed by glycerol medium without (c_1) or with (c_2) extra flavins. All experiments are in triplicate.

Figure 6. Maximum power density of MFCs boosted in different carbon sources without and with extra flavins extracted from polarization curves.

Schematic 1. Putative EET model for Gram-positive bacteria: where SLC is S-layer associated *c*-type cytochromes on the cell wall surface [22]; Cyt₁ is membrane *c*-type cytochromes, and Cyt₂, Cyt₃, Cyt₄ are all uncertain membrane-bound Fe(III)-reducing *c*-type cytochromes [16]; the EET pathway via flavins and acetate metabolism for *B. megaterium* spp. is depicted in the red-dotted frame.

Graphical abstract



Highlights

- A new EAM of Gram-positive *Bacillus megaterium* LLD-1 is reported.
- Riboflavin was actively secreted by LLD-1 and promotes the EET processes.
- LLD-1 can use exogenous flavins to promote the EET processes.
- Lactose and glycerol are the most efficient for LLD-1 in bioelectricity generation.